MIXED XYLANASE, β-GLUCANASE ENZYME PREPARATION, produced by a strain of *HUMICOLA INSOLENS*

New specifications prepared at the 61st JECFA (2003) and published in FNP 52 Add 11 (2003). An ADI "not specified" was established.

SOURCES

Produced by submerged fermentation of a non-pathogenic and non-toxigenic strain of *Humicola insolens.* The manufacturing process is composed of a fermentation process, a purification process, a formulation process and finally a quality control of the finished product. The cell mass and other solids are separated from the broth by vacuum drum filtration or centrifugation. Ultrafiltration and/or evaporation are applied for concentration and further purification. Residual production strain microorganisms are removed by germ filtration.

Active principles

beta-Glucanase and xylanase

Systematic names and numbers

beta-Glucanase: 1,3-(1,3;1,4)-beta-D-glucan 3(4)-glucanohydrolase
EC number: 3.2.1.6
CAS number: 62213-14-3

Xylanase: 1,4-beta-D-xylan xylanohydrolase
EC number: 3.2.1.8
CAS number: 9025-57-4

Reactions catalysed

Endohydrolysis of 1,3- or 1,4-linkages in beta-D-glucans
Endohydrolysis of 1,4-beta-D-xylosidic linkages in xylans

Secondary enzyme activities

Cellulase CAS No. 9012-54-8
Hemicellulase CAS No. 9025-56-3
Pentosanase CAS No. 9068-42-2
Arabinase CAS No. 75432-96-1

DESCRIPTION

Brown liquid.
The enzyme concentrate as manufactured is stabilized by the addition of sorbitol, glycerol, and potassium sorbate.

FUNCTIONAL USES

Enzyme preparation.
The preparation is used in beer brewing and in other fermentations to hydrolyse beta-glucans, pentosans, and other gums. This reduces the viscosity of the solution and thereby increases the filtration rate of both wort and beer, and haze is avoided. The enzyme is denatured and inactivated during wort boiling. The beer filtration process is likely to remove the denatured enzyme along with the other insoluble materials.

GENERAL SPECIFICATIONS

Must conform to the *General Specifications and Considerations for Enzyme Preparations Used in Food Processing* (see Volume Introduction)

CHARACTERISTICS

IDENTIFICATION

beta-Glucanase activity — The sample shows beta-glucanase activity.
See descriptions under TESTS

TESTS

beta-Glucanase activity

Principle:

Fungal beta-glucanase reacts with beta-glucan to release glucose or reducing carbohydrate which is determined as reducing sugar according to the Somogyi-Nelson method. One fungal beta-glucanase unit (FBG) is the amount of enzyme which according to the above outlined standard conditions, releases glucose or reducing carbohydrate with a reduction capacity equivalent to 1 μmol glucose/min.

Apparatus:

Thermostatic water bath (30°)
Spectrophotometer
Boiling water bath (≥ 100º)

Reagents and solutions

0.033 M Sørensen's phosphate buffer, pH 5.0: Dissolve 0.096 g disodium hydrogen phosphate dihydrate and 8.9864 g potassium dihydrogen phosphate in demineralised water and make up to 2000 ml.

Beta-glucan substrate: Dissolve 500 mg beta-glucan substrate in 40 ml 0.033 M Sørensen's phosphate buffer pH 5.0 with heating. Cool to room temperature and check the pH (5.0 ± 0.05). Make up to 50 ml in a volumetric flask.

Nelson's colour reagent, pH approx. 0.2-0.7: Dissolve 250.0 g ammonium molybdate tetrahydrate in 4500 ml demineralised water. Add 210 ml concentrated H_2SO_4 and 30 g disodium arsenate heptahydrate, mix and make up to 5000 ml with demineralised water. The reagent must be stored in the dark in a brown bottle.

Somogyi's copper reagent: Dissolve 175.5 g disodium hydrogen phosphate dihydrate and 200 g potassium sodium tartrate tetrahydrate in demineralised water and make up to 2500 ml. Add 500 g sodium hydroxide and 40 g copper sulphate pentahydrate and mix. Finally, add 900 g anhydrous sodium sulphate and make up to 5000 ml with demineralised water. The reagent must be stored in the dark in a brown bottle.

Glucose 2% stock solution: Dissolve 2.000 g anhydrous glucose in demineralised water and make up to 100 ml.

Glucose standard solution, 0.150 mg/ml: Dilute 1.5 ml of the glucose 2% stock solution and dilute to 200 ml with demineralised water up to 200 ml.

Enzyme standard: The enzyme standard is dissolved in demineralised water to produce a stock solution containing 2.5 FBG/ml. A range of further dilutions is made to establish a standard curve of enzyme activity containing 0, 0.02, 0.03, 0.06, 0.09, 0.10 FBG/ml.

Samples:

Enzyme samples are diluted to give activities in the range 0.03-0.09 FBG/ml covered by the standard curve.

Procedure:

Determination of activity relative to an enzyme standard (refer to table 1, columns A & B)

Pipette 1.0 ml enzyme solution into two tall (25 x 200 mm) test tubes (1 x sample (A) and 1 x control (B)). Heat in a water bath at 30° for 5 min. Add 1.0 ml beta-glucan substrate to the sample and 1.0 ml phosphate buffer to the control. Mix well and incubate at 30° for exactly 30 min. Add 2.0 ml copper reagent to both tubes and mix. Cover the tubes with glass balls and place them in a bath containing boiling water for 30 min. Cool in cold water to room temperature, remove the balls and add 2.0 ml colour reagent. Mix until any red precipitate has dissolved. Add 40 ml demineralised water and invert the tubes carefully three times.

Immediately measure the absorbance at 520 nm against a water blank. If the measurement is delayed more than 15 min invert the tubes once more before reading in the spectrophotometer.

Determination of absolute activity (refer to Table 1, columns A – E)

Analyse each sample in duplicate according to A, Table 1 using the procedure outlined above.. Use one control sample (B) and, in addition, perform a single determination on the substrate (C) a duplicate determination on the standard glucose solution (D) and a determination on a control sample (E). Measure the extinction at 520 nm and determine values for the following relationships:

Sample vs. control B (X)
Substrate vs. control E (Y)
Glucose standard vs. control E (Z)

The difference in glucose content between the sample and control B must not exceed 0.275 mg glucose/ml, otherwise the enzyme must be diluted further.

	Sample	*Control*	*Substrate*	*Glucose standard*	*Control*
	A	B	C	D	E
Enzyme solution	1 ml	1 ml			
Enzyme solution heated	30°	30°			
Substrate added	1 ml				
Phosphate buffer added		1 ml			
Incubated for 30 minutes	30°	30°			
Demineralised water			1 ml		
Substrate added			1 ml		
Glucose standard 0.15 mg/ml				2 ml	
Demineralised water					2 ml
Copper reagent added	2 ml	2 ml	2 ml	2 ml	2 ml
Boiling water for 30 minutes	Yes	Yes	Yes	Yes	Yes

Colour reagent added	2 ml	2 ml	2 ml	2 ml	2 ml
Demineralised water added	40 ml	40 ml	40 ml	40 ml	40 ml

Calculation:

Determination of activity relative to an enzyme standard

Plot a standard curve (∆OD as a function of FBG/ml. ∆OD is the value from tube A - the value from tube B for each enzyme dilution). Determine the activity of the samples relative to the standard curve.

$$\text{Activity} = S \cdot V / M = \text{FBG/g}$$

where:

S = FBG/ml read from the standard curve
V = sample volume
M = weight of sample in g

Absolute determination of activity

The absolute activity of a sample is calculated as follows:

$$\text{Activity} = G \cdot V / (M \cdot A \cdot L \cdot T) = \text{FBG/g}$$

where

G = glucose/ml sample (G2) - mg glucose/ml substrate (G1)
V = volume (ml) in which A is dissolved
M = molecular weight of glucose (0.180)
A = weight of sample in g
L = ml solution used for the analysis
T = reaction time in minutes (30)

Formulas for the calculation of glucose content:

$$G1 = Y \cdot Gc \cdot Gv/X$$
$$G2 = Z \cdot Gc \cdot Gv/X$$

where

Gc = mg glucose/ml for glucose standard
Gv = volume of glucose standard in assay

The results should be given to 3 significant figures, except for activities < 100 FBG/g which are given as 2.

Xylanase activity

Principle:

Determination of endoxylanase activity at ph 6.0. Xylanase samples are incubated with a remazol-stained wheat arabinoxylan substrate. Unconverted substrate is precipitated with ethanol. The intensity of blue colouring of the supernatant due to unprecipitated remazol-stained substrate degradation products is proportional to the endoxylanase activity. Endoxylanase activity in FXU (Farvet Xylan Units) is measured relative to an enzyme standard. The colour profile may vary from enzyme to enzyme. This can result in major dilution effects.

Apparatus:
Spectrophotometer
Thermostatic water bath

Centrifuge
10 ml plastic test tubes
Stopwatch

Reagents and substrates:

Phosphate buffer stock solution, 1.0 M: Dissolve 1210 g sodium dihydrogen phosphate monohydrate and 218.9 g disodium hydrogen phosphate dihydrate in demineralised water. Add 40 ml 4 N NaOH and make up to 10 l with water.

Phosphate buffer, 0.1 M, pH 6.00 ± 0.05: Take 1000 ml phosphate buffer stock solution and make up to 10 l with demineralised water. Adjust the pH to 6.0 ± 0.05 using either 4 N NaOH (reagent 6.2.4) or 2 N HCl

HCl, 2 M

NaOH, 4 M

Azo-wheat arabinoxylan substrate (Megazyme Ltd., Bray, Ireland) 0.5% w/v pH 6.00 ± 0.05: Weigh 0.500 g Azo-wheat arabinoxylan into a 150 ml beaker. Add approx. 90 ml 0.1 M phosphate buffer, heated to approximately 50°, while stirring. Continue stirring at 50°C for a further 20 min. Cool the substrate solution and adjust to pH 6.00 ± 0.05 before transferring to a 100 ml graduated flask. Fill to the mark with phosphate buffer.

Stop reagent: Pipette 6.65 ml 2 N HCl into a 100 ml graduated flask. Fill up to the mark with 99.9% ethanol.

Samples and standards:

Reference enzyme stock solution for standard curve: Calculate and accurately weigh approximately 1g FXU standard into a suitable graduated flask, dissolve in 0.1 M phosphate buffer by stirring for approximately 15 minutes and prepare at least 6 FXU standard solutions to give a range of activities between 0.2 and 1.4 FXU/ml. A sample of known activity is included at the beginning and the end of each analysis series or at least every 20 samples.

Samples:

Samples are diluted on the basis of their anticipated activity so that the activity of the final dilution is between. 0.4-1.4 FXU/ml. Results outside the working range may be used to assess the activity of the sample for the next run. Weigh dry or liquid samples directly into the flask. Granulated products may take a considerable time to dissolve.

Procedure:

Pipette 0.100 ml standard or sample solution into 10 ml test tubes, add 0.900 ml of substrate and mix. Incubate the tubes in a 50° water bath, 30 min. Add 5 ml stop reagent and mix for 10-20 sec. Leave the tubes to stand at room temperature, for 15-60 min and centrifuge at 4000 rpm for 15 min. Measure the absorbance of the supernatant at 585 nm within 20 min.

Calculation:

Use the measurements for the enzyme standards to plot a standard curve. The data may be fitted to a third order polynomial. Determine the corresponding enzyme activity values from the standard curve for the samples. The activity of each sample is calculated as follows:

$$\text{Sample activity (in FXU)/g)} = \frac{C \cdot F \cdot D}{W}$$

Where:

C = enzyme activity read from the standard curve (FXU/ml)
F = volume of sample
D = further dilution of sample (e.g. second or third dilution)
W = weight of sample weighed out